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PURIFICATION AND SOME PROPERTIES OF γ-CONGLYCININ IN SOYBEAN SEEDS

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Abstract—A 7S globulin (γ-conglycinin) which was one of four major antigenic components in soybean globulins was purified and found to be homogeneous on ultracentrifugation, disc electrophoresis, immunoelectrophoresis and disc electrofocusing by gel filtration, preparative-scale disc electrophoresis and two kinds of affinity chromatography. Subsequently, some physico-chemical properties of the protein were determined. The sedimentation coefficient, isoelectric point, MW and diffusion constant were 6·55S, pH 5·80, 104000 and $5{\cdot}80 \times 10^{-7}$ cm^2/sec, respectively. The protein was a glycoprotein which contained 5·49% total carbohydrate per protein. The protein did not aggregate and dissociate with a change of ionic strength from 0·1 to 0·5.

INTRODUCTION

Four major antigenically different components have been isolated from the reserve soybean proteins by Catsimpoolas *et al.* [1,2]. They were given the names glycinin, α-, β- and γ-conglycinin [3], glycinin was identical with the 11S globulin and γ-conglycinin with the 7S globulin isolated by one of us [4,5]. α-Conglycinin was a 2S soybean globulin component and β-conglycinin the major component of a crude 7S soybean protein prepared by Roberts and Briggs [6]. However, β-conglycinin, not γ-conglycinin, was proved to be identical with the 7S globulin (7S b-antigen) from the comparison of the immunological and the other several properties in the previous paper [7]. This paper describes the purification and some characteristics of γ-conglycinin.

RESULTS

Purification

Four purification steps were essentially employed for the purification of 7S a-antigen; (i) affinity chromatography on concanavalin A (Con A) Sepharose, (ii) gel filtration with Sepharose 6B, (iii) preparative-scale disc electrophoresis and (iv) affinity chromatography on 7S b-antibody Sepharose. In this paper, the antigenic protein which gave a precipitin arc band corresponding with γ-conglycinin in immunoelectrophoresis was temporarily named the 7S a-antigen.

Firstly by using a Con A Sepharose column according to the method of Kitamura *et al.* [8], the main contaminant, the 11S globulin, was eliminated from the crude 7S protein prepared previously to be free of the 2S ultracentrifugal contaminant by gel filtration with Sephadex G-100. The elution profile is shown in Fig. 1. All the 11S globulin eluted in peak A without absorption on the column as demonstrated by a single precipitin band reacted between the protein of peak A and monospecific 11S antiserum in immunodiffusion of Fig. 1. However, both 7S a- and b-antigen adsorbed to Con A Sepharose and eluted only in the latter peak B washing with 0·1M α-methyl-D-mannoside in the 0·5μ standard buffer from the results of immunoelectrophoresis in Fig. 1.

The concentration of 7S a-antigen in peak A of Fig. 1 was followed by gel filtration of Sepharose 6B. The elution pattern is shown in Fig. 2. Most 7S b-antigen with aggregated form in the ionic strength of 0·05 eluted in the former part of a single peak. 7S a-Antigen was concentrated in the latter shoulder part of the peak as shown in the immunoelectrophoretic result of Fig. 2.

Subsequently, 7S a-antigen was further concentrated by preparative-scale disc electrophoresis (Fig. 3). Peak A was riboflavin the catalyst of photopolymerization in

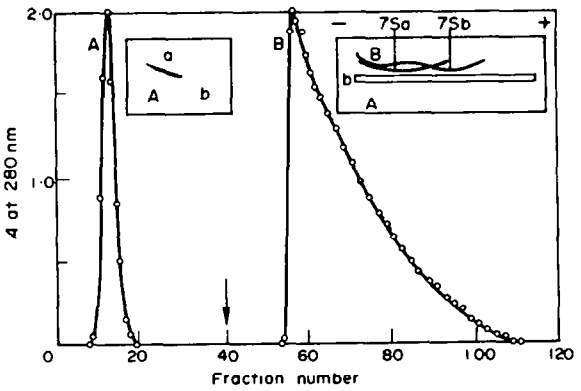

Fig. 1. Affinity chromatography of the crude 7S protein on a column of Con A Sepharose 4B. 7S a and 7S b were the immunoelectrophoretic bands of 7S a- and 7S b-antigen. The arrow indicates the addition of 0·1 M α-methyl-D-mannoside to the 0·5μ standard buffer. a; 11S-antiserum, b; 7S-antiserum.

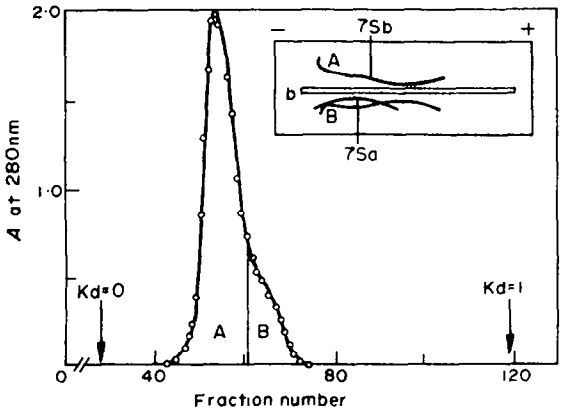

Fig. 2. Gel filtration of the crude 7S a-antigen with Sepharose 6B. *Kd* was the distribution coefficient in the gel filtration. 7S a and 7S b were the immunoelectrophoretic bands of 7S a- and 7S b-antigen against 7S antiserum (b).

the stacking gel and peak B was bromophenol blue the marker of mobility. 7S a-antigen was mostly concentrated only in peak C. Lastly, the slight contamination of 7S b-antigen in peak C was completely adsorbed to the affinity column of 7S b-antibody Sepharose.

Criteria of purity

A monospecific immunoelectrophoretic pattern of the protein was developed with the antiserum of a mixture of 7S a- and b-antigen (7S antiserum). The ultracentrifugal homogeneity of the preparation was also obtained. 7S a-antigen also showed a single band on disc electrophoresis. The mobility of the band was faster than either the 11S globulin or 7S b-antigen. The single electrophoretic band on disc polyacrylamide gel was also clearly stained for glycoproteins according to the method of Zacharius *et al.* [9]. A single band of 7S a-antigen in disc electrofocusing agreed with a minor band of soybean globulins focused toward the alkaline side [10]. The apex of the peak obtained by 7S a-antigen was found to be at pH 5·8. From these results, the 7S a-antigen was considered to be homogeneous.

Sedimentation behavior

It has been recognized that at least 2 7S components exist in soybean globulins from the difference of sedimentation behavior with the change of ionic strength [11].

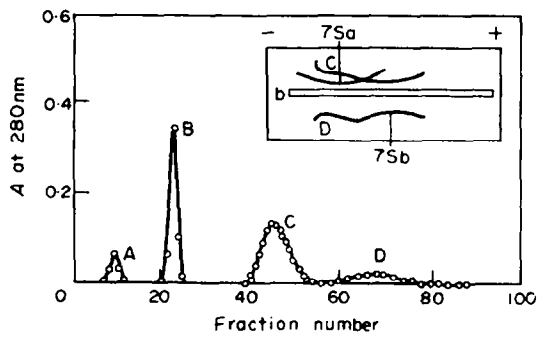

Fig. 3. Preparative-scale disc electrophoresis of part B in Fig. 2. 7S a and 7S b were the immunoelectrophoretic bands of 7S a- and 7S b-antigen against 7S antiserum (b).

The sedimentation constants of 7S a-antigen in 0·5 and 0·05 ionic strength were 6·39S and 6·43S, respectively at the protein concentration of 0·58%. Accordingly, 7S a-antigen did not undergo an association–dissociation reaction with a change of ionic strength from 0·1 to 0·5 as seen in the major 7S protein in soybean globulins [12], the 7S globulin.

Some physical characteristics

The constant extrapolated to zero concentration on the concentration dependence of the sedimentation coefficient was 6·55S. The diffusion coefficient of the protein was determined by two methods. The first was the immunological method of Allison and Humphrey [13]. Briefly, antigen and antibody are placed in troughs cut at a 90° angle in a uniform layer of agar. Precipitin occurred along a straight line inclined at an angle θ (50°) to the antigen trough. There is a close relation between θ, diffusion coefficient of antigen (Dg) and that of rabbit antibody ($Db = 3{\cdot}8 \times 10^{-7}$ cm^2/sec [14]).

$$\tan\theta = (Dg/Db)^{1/2} \quad (1)$$

Dg of 7S a-antigen in the 0·5μ standard buffer at 20° was calculated to be $5{\cdot}40 \times 10^{-7}$cm^2/sec. The value corrected with water as solvent was $5{\cdot}62 \times 10^{-7}$cm^2/sec ($D_{20,w}$).

Secondly, the constant of 7S a-antigen was obtained from sedimentation boundary curves by using an analytical ultracentrifuge. When the protein sample has the concentration dependence of the sedimentation coefficient (s), the diffusion coefficient (D) is given by the following equation (2)[15], $(A/H)^2 = 4\pi D \times (1 - z)t$, ($z < 0{\cdot}16$), where A is the area enclosed by the sedimentation boundary curve above its base line, H its maximum height and t the time measured from the start of centrifugation. In equation (2), z is calculated from equation (3) $z = 2r_0\omega^2 s^0 k c_0 \times (H/A)t$, and (4) $s = s^0(1 - kc)$, where c_0 is the initial concentration of the protein soln, ω the angular velocity of rotation, r_0 the radial position at which initially a sharp boundary is formed between solution and solvent, s^0 the value of s at infinite dilution and k a constant for the concentration dependence of the sedimentation coefficient. The value of k for 7S a-antigen was calculated to be 0·08 *dl*/g. The range of z was from 0·1 to 0·02. The diffusion coefficient was computed to be $5{\cdot}57 \times 10^{-7}$ cm^2/sec in the 0·5 μ standard buffer at a rotor speed of 11967 rpm and 20°. The value was corrected to be $5{\cdot}80 \times 10^{-7}$cm^2/sec with water as solvent.

By using the next equation (5) [16], $MW = RT/(1 - \bar{V}\rho)\, s/D$, the MW of 7S a-antigen was first determined. Where R is the gas constant ($8{\cdot}314 \times 10^7$ ergs/mol/degree), T absolute temp. ρ density of medium, s sedimentation constant ($6{\cdot}55 \times 10^{-13}$ sec), D diffusion constant ($5{\cdot}80 \times 10^{-7}$ cm^2/sec) and $\bar{V}$ partial specific volume. The value of the MW was calculated to be 102000 assuming that $\bar{V}$ was 0·725 ml/g.

Secondly, Yphantis' procedure [17] of sedimentation equilibrium in the centrifuge was used for the determination of the MW. About 106 min were required to attain equilibrium at 10209 rpm and 20°. Extrapolation to zero concentration of the protein gave the MW of 104000 using 0·725 ml/g as the value for the partial specific volume.

7S a-antigen was clearly shown to be a glycoprotein from the adsorption to Con A Sepharose (Fig. 1) and the staining of the band for glycoproteins after disc electrophoresis. The content of total carbohydrate was 5·49% per protein.

DISCUSSION

Catsimpoolas *et al.* [2] have isolated γ-conglycinin from the crude reserve soybean proteins in an immunologically and electrophoreticlly pure state by gel filtration with Bio-gel A-1·5 m and column chromatography with DEAE-Sephadex A-50. The protein was also isolated from a minor peak eluted after the main peak in the gel filtration of the same starting material with Sephadex G-150 [1]. However, we failed to isolate the protein according to both methods of Catsimpoolas since it was not possible to eliminate a slight amount of 7S b-antigen. However, 7S a-antigen activity was also only in the trailing edge of the main peak by gel filtration of the crude 7S protein [4] with Sephadex G-200 in the 0·5μ standard buffer. In this part, a 7S ultracentrifugal component was mainly found which lacked the ability of isomerization reaction with a change of ionic strength from 0·1 to 0·5 [18]. The 7S component must be identical with 7S a-antigen.

The comparatively strong activities of agglutinin and lipoxidase are retained in soybean globulins. Their MWs and isoelectric points are very similar to those of 7S a-antigen [19–26]. In addition, soybean agglutinin [21] is a glycoprotein with the 7S globulin [27,28]. However, 7S a-antigen had neither activity of agglutinin nor lipoxidase. No precipitin arc band by immunodiffusion was formed between the two proteins and monospecific anti-7S a-antigen serum. Moreover, the mobilities of each single band on disc electrophoresis were also different between 7S a-antigen and agglutinin, i.e. 7S a-antigen moved faster than agglutinin at pH 9·4. Catsimpoolas [29] has also reported that the lipoxidase is immunologically different from γ-conglycinin. Therefore, 7S a-antigen was completely different from the two proteins.

The content of 7S a-antigen was immunologically determined to be 3% in soybean globulin. On the other hand, the content of 7S b-antigen was 27·9% in soybean globulins. Therefore, although 7S a-antigen was one of major antigenic components in soybean globulins judging from the antigenic strength, the protein might be quantitatively a minor component of 7S ultracentrifugal components. The proportion of 7S area determined from ultracentrifugal pattern of soybean globulins in the 0·5 μ standard buffer was 34%. The ratio agreed approximately with the total proportion (30·9%) of 7S a- and 7S b-antigen determined immunologically.

EXPERIMENTAL

Materials. The 7S and 11S globulin were prepared as described previously [4,5,30]. The 7S globulin was further purified by preparative-scale polyacrylamide gel electrophoresis as occasion demanded. Purified soybean agglutinin was prepared by $(NH_4)_2SO_4$ fractionation using the initial partial purification of ref [31] and by column affinity chromatography with Con A Sepharose and Sephadex G-200 using the latter procedure described in ref [32].

Preparation of antisera. The details of preparation of antisera were carried out according to ref. [7]. The prepared antisera were as follows; 11S globulin (11S-antiserum), 7S a-antigen (7S a-antiserum), 7S b-antigen (7S b-antiserum) and 7S antigen antiserum (7S antiserum) which reacted against both 7S a- and 7S b-antigen.

Immunochemical methods. The details of immunoelectrophoresis, double gel immunodiffusion were as described in ref. [7]. A single diffusion technique was done for immunological quantitative determination of antigenic proteins using a glass tube (1 × 50 mm) according to the procedure of ref. [33].

Affinity chromatography and gel filtration. Two kinds of affinity chromatography for the purification of γ-conglycinin (7S a-antigen) were used. The first was Con A Sepharose gel (Pharmacia Fine Chemicals) in a glass column (2 × 45 cm) containing *ca* 140 ml of gel. The gel was equilibrated with the 0·5 μ standard buffer [34] (32·5 mM K_2HPO_4, 2·6 mM KH_2PO_4, 0·4 M NaCl, 0·01 M 2-mercaptoethanol, pH 7·6). The bound substances were eluted with 0·1M α-methyl-D-mannoside (Fluka AG, Chemische Fabrik) in the buffer. The other affinity column was packed with 7S b-antibody Sepharose 4B gel. 60 mg of purified 7S b-antibody was reacted with about 21 ml (6 g dry wt) of hydrated, washed CNBr-activated Sepharose 4B according to the Manufacturer's directions. About 50 mg of the antibody couplied to the gel judging from protein concn in washings obtained during the preparation procedures. A small column (1·5 × 12 cm) was prepared with 7S b-antibody Sepharose 4B and washed with 0·2 M Tris–HCl buffer containing 0·5 M NaCl, pH 8. The bound substances were eluted with 0·2 M glycine–HCl buffer containing 0·5 M NaCl, pH 2·8. The flow rate in both forms of affinity chromatography was 5·7 ml per hr. A long column (2 × 200 cm) packed with Sepharose 6B was used for gel filtration. The elution was carried out using 5 mM KPi buffer, pH 7·6 at a flow rate of 15 ml per hr. The *A* of each effluent in affinity chromatography and gel filtration was measured at 280 nm. Each fraction vol was 5·5 ml.

Disc electrophoresis. The details are described in ref. [7].

Disc electrofocusing. The details are described in ref. [10]. The staining of the electrofocused bands was performed with 0·5% amido black 10B dye in 7% HOAc soln after extensive washing of the gels with 12% CCl_3COOH in H_2O. The relative intensity of the stained bands was traced. Determination of pH range was done after slicing an unwashed gel and suspending the sliced gels in 1ml of H_2O overnight.

Other procedures. Sedimentation analysis, determination of carbohydrate and protein were performed as described in ref. [7]. The activity of soybean lipoxidase and agglutinin were assayed by the low EtOH method [35] and measuring the ability to agglutinate trypsinized rabbit erythrocytes [36], respectively.

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